1084-Plat
Transport Properties of a Prokaryotic CLC Transporter Assayed by Solid-Supported Membrane Electrophysiology
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EcClC (or ClC-Ec1), a prokaryotic member of the ClC family of channels and transporters of known structure, acts as a coupled chloride/proton antiporter. To investigate its electrogenic activity, proteoliposomes containing reconstituted EcClC were adsorbed on a solid-supported membrane (SSM) electrode. The application of concentration jumps of different anions results in robust transient currents with a selectivity sequence that agrees with previously reported measurements on planar lipid bilayers. The transient currents increase at acidic pH with an apparent pKa of 4.6.
The pH dependences of three mutations that are known to impair proton translocation while preserving chloride transport (mutations E148A, Y445A, and E203Q) have also been investigated. In agreement with previous results, E148A shows weak pH dependence. The transport impaired Y445A and the uncoupled E203Q present a shift in the apparent pKa towards less acidic values. Mutation E203Q, in addition, presents residual electrogenic activity at neutral pH values. Taken together, our results support the idea that chloride transport in the wild-type varies with pH mainly in response to titration of the external glutamate (E148).

1085-Plat
On the Mechanism of Gating Charge Movement of the Chloride/Proton Antiporter CLC-5
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Structural and functional studies identified two critical residues for the transport mechanism of CLC transporters; the so-called gating glutamate (E211 in ClC-5) that controls the access of the anions to the extracellular space and is critical for anion/proton coupling and the proton glutamate (E268 in ClC-5), likely the intracellular entry/exit point for protons. However, the mechanism of voltage-sensitivity of CLC transporters is still poorly understood.
Interestingly, it has been recently reported that the E268A mutant of the endosomal Cl-/H+ antiporter ClC-5, beside inhibiting steady-state transports, exhibits transient currents upon voltage steps to large positive voltages. These transient currents may offer the possibility to glean information on the molecular details of transport coupling and voltage-sensitivity. Here we studied the dependence of the transient currents on the extracellular and intracellular pH and Cl- concentration. We conclude that the transient currents represent the movement of an intrinsic gating charge followed by the voltage dependent binding of extracellular Cl-ions. In addition, we find that the gating glutamate mutation E211D abolishes stationary transport but displays transient currents which are shifted by ~ 150 mV compared to the proton glutamate mutation, identifying E211 as a major component of the voltage sensing mechanism of ClC-5.

1086-Plat
***Bath Salts*: A Synthetic Cathinone Whose Two Major Components Act Similar to Methamphetamine and Cocaine on the Human Dopamine Transporter**
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β-Keto-amphetamine analogs (synthetic cathinones) represent a new and rapidly growing class of abused substances. Members include cathinone (*khat*) and the mixture of mephedrone and MDPV (*bath salts),* which is increasingly popular in the United States. Similar to amphetamine and methamphetamine, cathinone and methcathinone work primarily at the dopamine transporter (DAT) as dopamine (DA) releasing agents and CNS stimulants. Theoretically, hundreds of synthetic cathinones are structurally possible, and more than a dozen analogs have been designated as illegal. With few exceptions, however, little is known about the pharmacology or mechanism of these new and powerful drugs. Furthermore, most cathinone analogs are unavailable in pure form for scientific investigation. We are synthesizing racemic mixtures and optical isomers of synthetic cathinones, some of which are already on the clandestine market, to investigate their pharmacology and mechanism of action. Electrophysiological studies of *bath salts* on hDAT-expressing frog oocytes show that one component, mephedrone, has an electrical signature similar to methamphetamine, while another component, MDPV, has the electrical signature of cocaine. In particular, 10 μM mephedrone elicits an inward current at −60 mV that persists long after the drug is removed externally, similar to the *molecular stent* mechanism described for S(+)Amphetamine (Rodriguez-Menchaca et al., British J Pharmocology, 2011). MDPV on the other hand elicits an outward current under similar conditions, indicative of a blocking agent similar to cocaine. We have verified MDPV block of hDAT in ^{3}H-DA uptake experiments. Our results indicate that *bath salts* contain a DA releasing agent and a DA reuptake inhibitor. The two drugs have different kinetics and rather than cancel each other they would exacerbate the effect of either drug applied alone.

1087-Plat
Direct Observation of Conformational Exchange in the Small Multidrug Resistance Transporter EmrE
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Small multidrug resistance (SMR) transporters provide an ideal system to study the minimal requirements for active transport across a membrane. EmrE is an E. coli SMR transporter that exports a broad class of polyaromatic cation substrates, thus conferring resistance to drug compounds matching this chemical description. As a secondary active antiporter, EmrE drives the uphill export of each substrate molecule by coupling it to the downhill import of 2 protons across the inner membrane. EmrE is proposed to function via a single-site alternating access model. In this well-established model, transporters are inherently dynamic proteins, converting between inward- and outward-facing conformations in order to move substrate molecules across a membrane barrier. There is general agreement that the minimal functional unit is an EmrE homodimer, but a great deal of controversy remains regarding its structure, topology, and detailed mechanism. We have used a combination of NMR and FRET experiments to directly follow the kinetics and structural changes occurring during individual steps in the transport cycle. Our results reveal that EmrE forms an antiparallel homodimer and exchanges between inward- and outward-facing states at a rate of 5 s-1 when bound to the substrate tetraphenylphosphonium. Furthermore, the inward- and outward-facing states are identical except that they have opposite orientation. These findings reconcile the controversial asymmetric EmrE crystal structure with the functional symmetry of residues in the active site and have important implications for the energetics of proton-driven coupled antiport.

1088-Plat
Mechanistic Investigations into the Multi-Drug Resistance Transporter, EmrE
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EmrE, an *E. coli* small multidrug resistance transporter, exports a broad range of toxic polyaromatic cations, thus imparting resistance to drug compounds of this type. According to the proposed single-site alternating access model of antiport, EmrE converts between inward- and outward-facing structures during the transport cycle, using the import of two protons across the inner membrane to drive the export of one substrate molecule. Interconversion between the inward- and outward-facing conformations must occur only in the substrate-bound state in order to achieve coupled antiport. Our research focuses on this coupling between substrate binding and conformational exchange.
Conformational exchange between inward- and outward-facing states under substrate-bound conditions is the key step to efflux. We have directly monitored conformational exchange in tetraphenylphosphonium+-bound EmrE using ZZ-exchange NMR spectroscopy. This gives a rate constant of about 5 sec^{-1} for TPP^{+}-bound EmrE converting from an inward- to outward-facing conformation or vice versa. Stopped-flow kinetics were employed to measure the ligand on/off rates. The inward- and outward-facing states are identical in this system. This facilitates analysis of all the microscopic steps in the transport cycle, and has significant implications for the energetics of the transport process.
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1089-Plat
Mechanism and Regulation of Urea Permeation in a Mammalian Urea Channel
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To maintain constant fluid volume and osmolarity in the face of infrequent access to water, terrestrial animals accumulate high concentrations of urea in the kidney interstitium to allow the reabsorption of water. This mechanism is dependent on the facilitated diffusion of urea through members of a family of

integral membrane proteins known as urea transporters (UTs). While the structure of a bacterial homolog was solved previously, the bacterial UT transports urea much slower than the mammalian ones and its similarity to the mammalian proteins remained unclear. In addition, little was known of the mechanism of permeation. To answer these questions, we have solved the first structure of a mammalian urea transporter at 2.4 Å, and probed the energetics of urea permeation with a combination of molecular dynamics simulations, functional characterization of mutants and co-crystallization with urea analogs. Similarities with the bacterial UT suggest that many features of the structure are broadly conserved across the UT family, including a trimeric assembly, and the presence of a channel-like, continuous permeation pathway through each monomer. The functional and computational studies revealed a large energy barrier at the center of the permeation pathway, whose presence may hint towards a possible gating mechanism.

Platform: Fluorescence Spectroscopy

1090-Plat

Unraveling Folding Pathways and Kinetics Transition of T4 Lysozyme with High Temporal Resolution by Single Molecule FRET

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Protein folding is directly connected to protein function, and miss folding events could lead to disease. Currently, one of the challenges of understanding protein folding is to determine the number of meta stable or excited intermediate states and their corresponding kinetic pathways during folding transitions given the large number of possible accessible conformers. Therefore, we combine the power of single molecule FRET, with site specific labeling, filtered fluorescence correlation spectroscopy (fFCS), multiparameter fluorescence detection (MFD) and ensemble time correlated single photon counting to determine the folding and unfolding kinetics and the corresponding reaction pathways of T4 Lysozyme (T4L) under various chemical denaturation conditions (Urea, GdmCl, and pH) with sub microsecond resolution.

MFD, a single molecule technique, allows the direct observation of populations; even when low populated (<10%). fFCS, a recently developed modification of the standard FCS, uses the fluorescence lifetime and anisotropy decays to filter the signal and properly weight the contribution of each photon to the corresponding population in an heterogeneous solution. The weighted time dependent signal with specific spectral window and polarization is correlated to obtain all possible auto-correlation and cross-correlation curves. Of great utility is the cross-correlation curve because it resolves, with maximum contrast, the anticorrelated behavior of the interconversion between populations.

Using the afore mentioned toolbox, we observe that each denaturant results in different folding and unfolding pathways in T4L. Furthermore, the addition of surfactant (Tween 20) changes dramatically the reaction coordinate in the folding and unfolding energy landscape even in the same denaturant conditions. One of the major differences observed is that pH denaturation showed the accumulation of an intermediate state where most likely the N terminal domain is partially unfolded while the C terminal domain remains folded.

1091-Plat

Correcting for Artifacts from Spectral Cross-Talk and Imperfect Detection Volume Overlap in Dual-Color Fluorescence Cross-Correlation Spectroscopy

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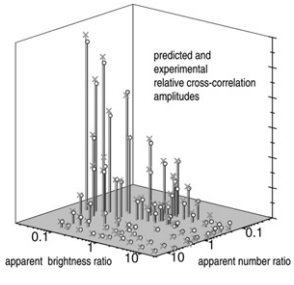
Dual-color fluorescence cross-correlation spectroscopy (dcFCCS) allows to quantitatively assess the interactions of mobile molecules labeled with distinct fluorophores.

One major artifact risk in dcFCCS is a false-positive or overestimated cross-correlation amplitude arising from spectral cross-talk. Cross-talk can be reduced or prevented by fast alternating excitation, but the technology is not easily implemented in standard commercial setups.



We devised an experimental strategy that does not require specialized hard- and software for recognizing and correcting for cross-talk in standard dcFCCS. Another major artifact risk in dcFCCS is a false-negative or underestimated cross-correlation amplitude arising from an imperfect detection volume overlap. Samples based on fluorophores conjugated to oligonucleotides that have been traditionally used for dcFCCS calibration typically suffer from incomplete labeling. Using these samples, it is difficult to attribute a reduced cross-correlation amplitude to the imperfection of the calibration sample or to that of the setup. We therefore developed a new cross-correlation calibration standard with a predictable degree of labeling.

1092-Plat

Confined Dynamics and Membrane Domain Features Revealed by Image Correlation Spectroscopy Toolkit

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The modern model of the cell membrane posits that it is spatially heterogeneous with domains that restrict molecular transport. Lipid rafts, or microdomains, are one such membrane domain that are enriched in sphingolipids, and function to sequester proteins in transient complexes that are believed to be a few 10s to 100s of nanometers in size. Membrane proteins may also be sequestered by the membrane proximal actin cytoskeleton. Here we show that image correlation based techniques, applied to standard laser scanning or TIRF fluorescence microscopy image series, can be used to discriminate between the different mechanisms of confined diffusion. We plot the diffusion time lag versus the waist of the correlation functions at a given time lag, to obtain the Mean Square Displacement (MSD) from the correlation function width, which is analogous to the previously described FCS diffusion law for variable beam radii. The ICS approach has the advantage of being based on image analysis without the need to vary the beam size. Furthermore, we show how one can extract from the correlation function data the characteristic parameters of the system such as domain size, density, diffusion coefficients and partition rates. To verify the validity of this tool, we performed simulations of confined diffusion in meshwork and microdomains where we varied the domain size (10–1000 nm radius), density (up to 10 % area coverage), confinement probability and diffusion coefficients (0.002–0.1 μm2/s). We used the simulations to establish the limits due to the spatio-temporal sampling and noise. We applied this analysis to the study of dynamics of membrane proteins known to be raft associated: GPI-GFP and Cholera Toxin Subunit B, and verified that our tools can detect changes in the confinement parameters following the application of drugs that disrupt rafts.

1093-Plat

Tracking of Single Microvilli to Study Regulation of the Intestinal Phosphate Transporters

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Intestinal phosphate (Pi) uptake is one of the key mechanisms of systemic Pi homeostasis. Modulation of intestinal Pi transport has been recently recognized as an important target in prevention of hyperphosphatemia and the associated cardiovascular complications in Chronic Kidney Disease. Intestinal Pi absorption is mainly mediated by the type IIb sodium-phosphate co-transporter NaPi2b which is expressed in the intestinal apical microvilli. Recent evidences show that agonists of the LXR nuclear hormone receptor inhibit intestinal NaPi transport activity, NaPi2b protein and mRNA abundance, and decrease serum Pi level [1]. However, the detailed mechanisms of how LXR decreases intestinal NaPi transport activity, including the role of changes in membrane cholesterol or PDZ interacting proteins, remain to be determined.

In order to study these processes at the molecular level we apply the Modulation Tracking nanoimaging method [2] to microvilli of live cells expressing fluorescently tagged NaPi2b. This technique generalizes the principles of Single Particle Tracking to allow the imaging of extended subcellular structures. Tracking the single microvilli we directly measure their motion, while we also image NaPi2b distribution along the membrane and perform Fluorescence Correlation Spectroscopy techniques (RICS, N&B) in steady state, even if the microvilli are moving. Different dynamic properties, including motility of the microvilli and protein diffusion and clustering, are monitored under stimulation to get insights into the process of intestinal transporters regulation.

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